Simple Mucin-Type Carbohydrates in Oral Stratified Squamous and Salivary Gland Epithelia

Ulla Mandel, Ole William Petersen, Henning Sørensen, Poul Vedtofte, Sen-Itiroh Hakomori, Henrik Clausen, and Erik Dabelsteen

Departments of Oral Diagnosis (UM,HC,ED) and Oral and Maxillofacial Surgery (PV), Royal Dental College, Structural Cell Biology Unit, Anatomy (OWP), The Panum Institute and Blood Bank (HS), University Hospital, Copenhagen, Denmark; and The Biomembrane Institute and University of Washington (S-IH), Seattle, U.S.A.

Simple mucin-type carbohydrate antigens, T, Tn, and sialosyl-Tn, have been found to be good markers of malignant transformation in several epithelial tissues as a result of incomplete synthesis with precursor accumulation. The T, Tn, and sialosyl-Tn antigens represent the initial, most immature glycosylation of serine and threonine amino acids of proteins. In normal adult cells these structures are generally masked by addition of further saccharides to form more complex structures. We analyzed simple mucin-type carbohydrates in human labial stratified squamous and minor salivary gland epithelia in order to define the glycosylation pattern in normal cells in relation to epithelial differentiation and maturation. A panel of monoclonal antibodies with well-characterized specificity for T, Tn, sialosyl-Tn and the histo-blood group H and A variants hereof were used in immunohistology of sections from 30 individuals with known ABO, Lewis, and secretor status. In stratified epithelium the sialylated T structure was confined to cell membranes of immature basal cells, whereas the H and A variants were observed on cell membranes of more mature parabasal and spinous cell layers. Furthermore, superficial spinous cells produced a fine granular cytoplasmic staining for Tn and sialosyl-Tn antigens. In minor salivary glands mucous cells expressed Tn and sialosyl-Tn as well as the H and A variants in the area of the nucleus, whereas T and the H variant were found in duct cells and unsubstituted T antigen in myoepithelial cells. These results indicate that incomplete synthesis, i.e., deletion of sialyltransferases and/or histo-blood group ABH transferases, may result in accumulation of T, Tn, and sialosyl-Tn antigens in oral epithelia, thus offering a baseline for further studies of changes in premalignant and malignant oral epithelia. *J Invest Dermatol 97:713–721, 1991*

Much interest has been devoted to simple mucin-type (O-linked to proteins) carbohydrate antigens, the T (Galβ1 → 3GalNAcα1 → O-Ser/Thr), Tn (GalNAcα1 → -O-Ser/Thr), and sialosyl-Tn (NeuAcα2 → 6GalNAcα1 → O-Ser/Thr) antigens, because these antigens generally are strongly expressed in carcinomas, but only limited expressed in normal adult cells [1]. Simple mucin-type structures are normally masked in human cells and secretions due to sialylation and/or chain elongation and branching by the addition of other sugar residues, e.g., histo-blood group ABH determinants (see Fig 1).

The unmasked Thomsen-Friedenreich (T) antigen [2,3] was first discovered on cell membranes of normal erythrocytes, explaining the panagglutination phenomenon [4]. In various cancer cells presence of unmasked T antigen was initially suggested by Vaith and Uhlenbruch [4] and Springer et al [5], and this observation was subsequently supported by many other investigators [1,6–19]. Lectins and polyclonal antibodies traditionally used to detect T antigen do not specifically define the mucin-type T structure, but may also in addition to other glycoconjugates having a terminal galactose residue recognize the β-linked disaccharide, Galβ1 → 3GalNAcβ1-R [7,9,20,21]. Monoclonal antibodies (MoAb) have now been prepared that may distinguish these variants of the T disaccharide [9,20,22–24], thus permitting more detailed studies of the expression of the structures.

The Tn antigen that is the biosynthetical precursor to T was first recognized by Springer et al [25] as a widely expressed tumor-associated antigen. Similarly sialosyl-Tn antigens were associated with neoplastic transformation [26] and this has been confirmed with monoclonal antibodies [17,19,22,27–31].

The general biosynthetic pathway of "mucin-type" glycosylation has been established [32,33] (see Fig 1), but information regarding the biosynthesis in relation to normal cyto- and histo-differentiation is lacking. Stratified squamous epithelium of the oral mucosa offers an excellent system with a well-defined differentiation and maturation pattern with which changes in glycosylation may be related [34,35]. In the present study we show that simple mucin-type carbohydrates are used as a "carrier" of ABH variants in stratified squamous epithelium, and that these carbohydrates are sequentially synthesized in relation to cell maturation under the control of histo-blood group status. The unsubstituted Tn and sialosyl-Tn antigens were almost exclusively found intracellularly in both stratified and salivary gland epithelia.

MATERIAL AND METHODS

Tissue Four-millimeter punch biopsies of normal human oral mucosa including minor salivary glands were taken in the upper lip

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Reprint requests to: Ulla Mandel, Department of Oral Diagnosis, Royal Dental College, Norre Alle 20, DK-2200, Copenhagen N, Denmark.

Abbreviations:
FITC: fluorescein-isothiocyanate
MoAb: monoclonal antibody
PBS: phosphate-buffered saline, pH 7.2

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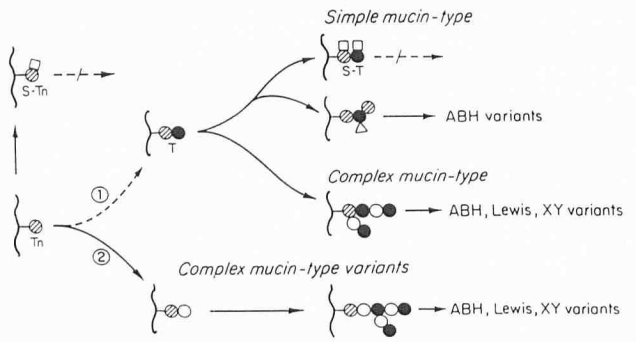

Figure 1. Biosynthetic pathway of simple mucin-type glycosylation. The initial glycosylation of serine and threonine amino acids of protein with a N-acetylgalactosamine constitutes the Tn antigen (GalNAcα1 → O-Ser/Thr). This structure may extend with a galactose residue forming the T-antigen or, alternatively, substituted with a sialic acid resulting in the sialosyl-Tn antigen. Another possibility is that Tn or T may be extended with glucosamine residues giving rise to the complex-mucin type variants with build-up of a polylactosamine branched or unbranched oligosaccharide structure that may express ABH, Lewis, and X/Y antigens. In stratified epithelium it appears from the present study that the T-antigen is apparent in immature basal cells as a sialosyl-substituted structure. In more mature parabasal and spinous cell layers ABH variants are expressed in secretors. Nonsecretor individuals expressed sialosyl-T in all cell layers. The most simple structures, Tn and sialosyl-Tn, were weakly expressed in the upper spinous cell layers, but these antigens were only found intracellularly in the cytoplasm.

close to the sulcus alveolo-buccalis in the canine region from 30 young healthy volunteers (18 female, 12 male) after informed consent (Table I). Biopsies were taken under local infiltration anaesthesia with 2% lidocaine with adrenalin and divided in two. Half of the tissue was fixed in 10% neutral buffered formalin for 24 h at room temperature and embedded in paraffin. The other half was quick-frozen in iso-pentan, precooled on dry ice, and stored at −80°C. ABO and Lewis blood groupings were determined on erythrocytes and secretor status tested on saliva at the Blood Bank, University Hospital (Copenhagen) by using mouse MoAb to Le^a, Le^b, and A, human typing sera to B and Ulex europaeus I for H (defining blood group O) (all reagents from Biotest, Hamburg, BRD). Secretor status is defined by presence of ABH blood group substances in saliva. Secretors secrete these substances, whereas nonsecretors do not. This phenomenon is controlled by the secretor gene encoded α1-2 fucosyltransferase (H-transferase), i.e., secretors express this transferase, which produce H-substrate for the AB blood group transferases. The lack of transferase activities in nonsecretors leads to lack of substrate for the AB transferases thus lack of AB substances in saliva [36]. The A_1A_2 distinction was done by routine procedures by using Dolichus Biflorus.

Table I. Distribution of ABO, Lewis, and Secretor Status of Individuals

	Lewis group	
ABO Group	Le^{a+b-} Nonsecretor	Le^{a-b+} Secretor
B	0	1
A_1	0	10
A_2	1	6
0	3	9
Total	4	26

Antibodies A panel of MoAb with well-defined specificity for derivatives of simple "mucin type" carbohydrates linked through the hydroxyl group of serine or threonine of proteins was used. Antibodies, their isotype, and reference for the procedure for their generation, isolation, and specificity are given in Table II. The MoAb to A, H, and T were characterized by using glycolipids with the type 3 or 4 chain structures, and only in the case of HH8 (T) has it been possible to test the appropriate type 3 chain glycoprotein antigen (asialo-glycophorin) [20]. The MoAb HH5 (mucin-type A) and MBr1 (mucin-type H) was, however, found to bind ovarian cyst glycoprotein indicating specificity for these mucin-type structures.* We have applied the following arguments when interpreting antibody reactivity with presence of the mucin-type antigen. *MoAb HH8* (T): HH8 also reacts with the glycolipid antigen galactosyl-A, a histo-blood group A associated carbohydrate structure, but this structure is exclusively present in tissues of blood group A individuals and not O and B, in contrast to mucin-type T [20]. *MoAb MBr1* (mucin-type H): MBr1 reacts with both H type 3 and 4 determinants on glycolipids; however, H type 4 is only found on lipids and generally not believed to be found on protein [34]. It is therefore

* Clausen H, Kabat E, Hakomori S (personal communication).

Table II. Structures of Carbohydrate Epitopes Investigated in the Present Study[a]

Antigen	Structure	Defined by MoAb (isotype)
Tn	GalNAcα1→O—Ser/Thr	BM8 (IgG_{2A})[b] TKH6 (IgM)[c]
Sialosyl-Tn	NeuAcα 2 ↓ 6 GalNAcα1→O-Ser/Thr	TKH2 (IgG_1) [27] B72.3 (IgG_1) [28]
T	Galβ1→3GalNAcα-R	HH8 (IgM) [20]
H	Galβ1→3GalNAcα/β-R 2 ↓ Fucα1	MBr1 (IgM) [62,63] HH14 (IgM)[b]
A	GalNAcα1→3Galβ1→3GalNAcα/β-R 2 ↓ Fucα1	HH5 (IgM) [64]

[a] See *Materials and Methods* for detailed discussion of specificity of MoAb to T, H, and A structures.
[b] Clausen H, Hakomori S (unpublished results).
[c] Kjeldsen T, Hakomori S, Clausen H (unpublished results).

unlikely that reactivity in deparaffinized sections should be due to glycolipids [37]. *MoAb HH5* (mucin-type A): HH5 reacts with A type 3 and 4 chain determinants on glycolipids as well, but a similar argument as for MBr1 may apply. All antibodies were used as hybridoma culture supernatants except MBr1, which was available as ascites.

Immunohistology Sections were cut from paraffin or frozen blocks at a thickness of 5 μm and mounted on gelatin-coated slides. All antibodies were tested on sections from both frozen and paraffin blocks. Neighboring hematoxylin-eosin stained sections helped in identifying positive structures. Antigens were detected in the tissue by a double-layer immunofluorescence technique. Paraffin sections were deparaffinized, brought to water, and washed in phosphate-buffered saline (PBS), pH 7.2. Frozen sections were fixed in acetone for 10 min at 4°C. Sections were incubated with MoAb in a moist chamber for 18 h at 4°C and then washed 3 times in PBS (5 min each). Fluorescein-isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin absorbed with human serum (Dakopatts, Denmark) was applied for 40 min. The sections were again washed and mounted in glycerol containing p-phenylene-diamine [38]. Paraffin sections subjected to sialidase treatment were preincubated for 2.5 h (frozen sections were incubated for 30 min) at 37°C with neuraminidase from *C. perfringens* type X (Sigma), 0.1 units sialidase per ml in a 0.1 M sodium-acetate buffer at pH 5.5, and stained with the MoAb. Slides were examined in a Zeiss fluorescence microscope with epi-illumination. The microscope was equipped with FITC interference filters (The Optical Laboratory, Lyngby, Denmark) and a 50 W xenon lamp. Control reactions consisted of 1) staining with conjugate alone, 2) substitution of the primary hybridoma supernatant with supernatant from the myeloma cell line SP2 used for hybridization, and 3) substitution of primary antibody with antibody of other specificity but with same isotype.

Double Immunostaining This was performed to identify positive structures. The sections were incubated for 5 min at room temperature in PBS supplemented with 10% goat serum (PBS-G) [39]. Sections were next incubated overnight at 0–4°C in PBS-G containing MoAb HH8 against T, rinsed 2 times 5 min in PBS followed by 1 h incubation at room temperature in PBS-G containing FITC-conjugated goat-anti-mouse IgM (diluted 1:100; catalog number M31501, Medac, Hamburg, BRD). Sections were then rinsed twice in PBS and further incubated with the second sequence of antibodies. Sections were incubated for 30 min at room temperature in PBS-G containing MoAb HHF-35 against α- and γ smooth muscle isoforms of actin (Enzo, NY [39–41]). Sections were then rinsed twice for 5 min at room temperature in PBS, and further incubated 30 min at room temperature in PBS-G with Texas Red conjugated goat anti-mouse IgG (catalog number 1070-07, Southern Biotechnology Associates, Inc., purchased from AB Kemila, Sollentuna, Sweden). Incubations in which the second antibodies were switched to the other sequence served as controls. The sections were mounted in 20 μl Fluoromount-G (Catalog number 100, Southern Biotechnology Associates, Inc.) containing 2.5 mg/ml freshly prepared n-propyl gallate (Sigma).† Preparations were observed and photographed in a Leitz Dialux 20 equipped with epifluorescence and a HBO 50 W high pressure mercury lamp. The following Leitz filter blocks were used: for Texas Red, set N2; for fluorescein, set L3. All observations were made using a ×40 PL APO objective.

RESULTS

Histologically all biopsies showed a normal stratified non-keratinized epithelium as well as minor salivary glands consistent with previous descriptions [42,43]. Staining intensity and distribution showed some variation from individual to individual even within the same genetical blood group status, but a distinct staining pattern (Table III) was clearly observed with each antibody in individuals of same blood group.

Stratified Squamous Epithelium Generally, staining of fixed, paraffin-embedded sections and fresh acetone-fixed frozen sections gave similar results. In the following the results refer to staining of paraffin-embedded sections unless specifically indicated that frozen sections differed.

The MoAb to the mucin-type T antigen (HH8) did not stain untreated sections. However, after neuraminidase treatment HH8 stained cell membranes of the basal cell layer homogeneously, indicating the presence of T antigen substituted with sialic acids (Fig 2*a*) [20]. This pattern was consistent in secretors irrespective of ABO blood groups, whereas nonsecretors stained cell membranes in the entire epithelium (Fig 2*d*). In frozen sections, occasionally a few single cells in the basal cell layer of the epithelium at the base of rete pegs stained with HH8 without pretreatment with neuraminidase irrespective of blood group and secretor status (Fig 3).

Anti-mucin-type H MoAb (MBr1 and HH14) stained cell membranes of cell layers above the basal cells in sections of secretors (Fig 2*b*). Both antibodies, although more pronounced for HH14, labeled fewer cells in histo-blood group A_1 individuals as compared to A_2, B, and O individuals giving a patchy distribution rather than the otherwise observed homogeneous pattern. This difference in staining intensity between MBr1 and HH14 was interpreted as lower affinity and more restricted specificity of the HH14 MoAb as has been demonstrated in immunochemical studies using H-active glycoli-

† Rønnow-Jessen et al (in preparation).

Table III. Tissue Distribution of Mucin-Type Carbohydrate Antigens

		Labial Mucosa					Minor Salivary Gland			
				Spinosum						
	Structure	Basal	Parabasal	Lower	Upper	Intermediate and Superficial Layers	Mucous Cells	Serous Cells	Duct Cells	Myoepithelial Cells
Secretor	Tn	−	−	+/−	+	+	+	−	−	−
	S-Tn	−	−	−	+/−	+/−	+	−	−	−
	T	+[a]	−	−	−	−	−	−	+	+
	S-T	+	−	−	−	−	−	+	+	+
	H	−	+	+	+	+	+	+	+	−
	A	−	−	+	+	+	+	−	−	−
Nonsecretor	Tn	−	−	+/−	+	+	+	−	−	−
	S-Tn	−	−	−	+/−	+	+	−	−	−
	T	+[a]	−	−	−	−	−	−	+	+
	S-T	+	+	+	+	+	−	+	+	+
	H	−	−	−	−	−	−	+	+	−
	A	−	−	−	−	−	−	−	−	−

[a] Few positive cells.

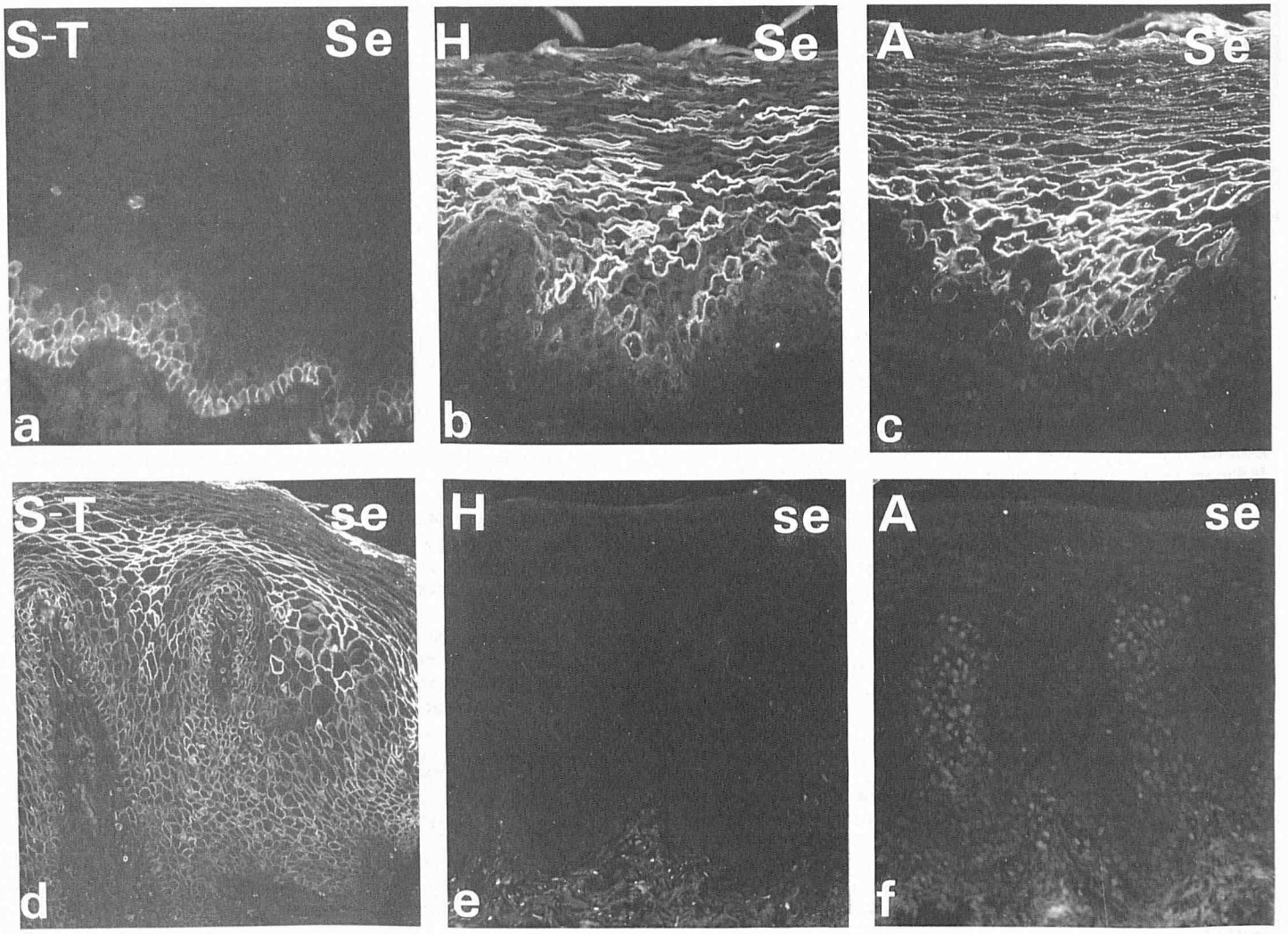

Figure 2. Immunohistologic staining of nonkeratinized stratified squamous epithelium of human oral mucosa. Simple mucin-type antigens in a blood group A_1, Le^{a-b+}, secretor individual (Se) *(a,b,c)* and in two Le^{a+b-}, nonsecretor individuals (se) of blood group O *(d,e)* and A_1 *(f)*. *(a)* Sialosyl-T (T detected after neuraminidase treatment) on cell membranes of basal cell layer in secretor versus *(d)* all cell layers in non-secretor (neuraminidase treated sections stained with MoAb HH8, ×120). *(b)* H on cell membranes of more mature parabasal and spinous cell layers in secretor in contrast to *(e)* no reaction in nonsecretor (stained with MoAb MBr1, ×120). *(c)* A on cell membranes of spinous cell layers in secretor, and *(f)* no staining in nonsecretor (×120).

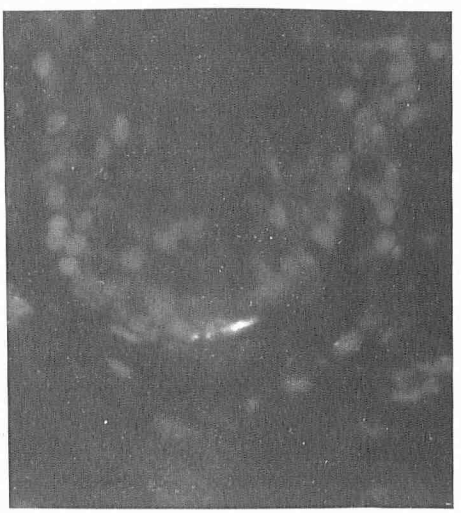

Figure 3. Immunohistologic staining of nonkeratinized stratified squamous epithelium of human oral mucosa. T on single basal cell at the base of a rete peg (blood group O, Le^{a-b+}, secretor individual stained with MoAb HH8, ×300).

pids.‡ In general, neither antibodies labeled nonsecretors (Fig 2*e*), although MBr1 stained a few sporadic parabasal cells in frozen sections of a few nonsecretors.

The anti-mucin-type A MoAb (HH5) stained cell membranes in spinous cell layers of A_1 secretors (Fig 2*c*) with considerably weaker reaction in A_2 secretors. In deeper cell layers HH5 in addition produced a fine granular cytoplasmic staining, and this was most pronounced in the A_2 secretors. Nonsecretors did not stain at all (Fig 2*f*).

The distribution of sialosyl-T (T antigen detected after neuraminidase treatment) and mucin-type H appeared not to overlap as evaluated by serial sections. The differences between secretors and nonsecretors showed a reciprocal relationship, indicating that the T disaccharide in secretors were converted to H. In addition, the lesser staining with anti-mucin-type H MoAb in A_1 secretor individuals was complemented by anti-A binding.

The most immature structures Tn (MoAb BM8 and TKH6) and sialosyl-Tn (MoAb TKH2 and B72.3) were with varying intensity seen as fine granules in the cytoplasm of superficial cell layers with all antibodies irrespective of blood group and secretor status (Fig 4*a,b*). Cell membrane staining was only rarely seen. Staining by

‡ Clausen H, Hakomori S (unpublished).

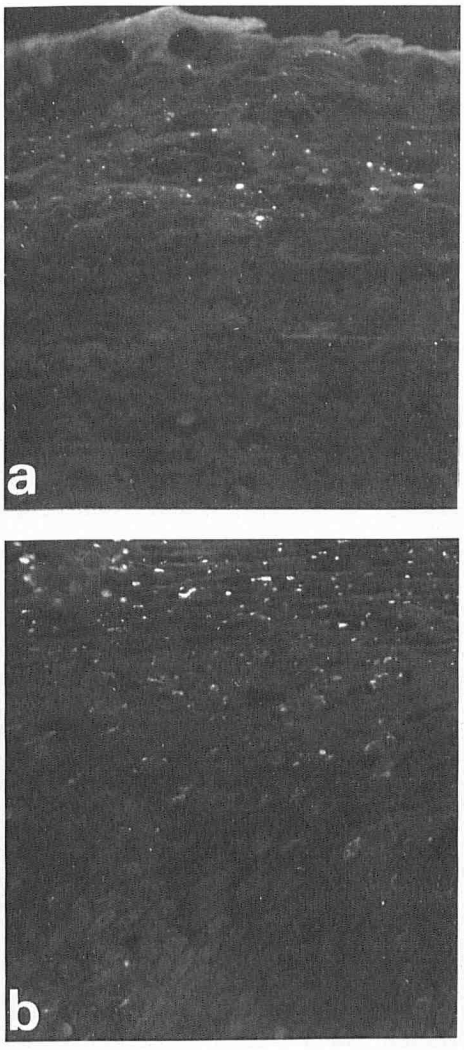

Figure 4. Immunohistologic staining of nonkeratinized stratified squamous epithelium of human oral mucosa. *(a)* Tn in cytoplasm of superficial spinous cells (blood group O, Le^{a-b+}, secretor individual stained with MoAb BM8, ×300). *(b)* Sialosyl-Tn in same location as Tn, although weaker staining (same individual stained with B72.3 ×300).

TKH2 and B72.3 for sialosyl-Tn was similar to Tn, however, weaker and only found in about half of the individuals. The sialosyl-Tn staining was abolished by pretreatment of sections with neuraminidase.

Glandular Epithelium of Minor Salivary Glands The epithelium of minor salivary glands comprises several distinct cell types with different functions. Acini in small salivary glands mainly consist of mucous cells, but serous cells may also be present. Small intercalated ducts or larger excretory ducts are numerous. Myoepithelial cells are associated with secretory endpieces or rarely with intercalated ducts, and require special stains for demonstration in light microscope [43].

Detection of mucin-type antigens in salivary glands was found to vary with fixation more profoundly than in stratified epithelium. Generally, less staining both in intensity and distribution was found in formaldehyde paraffin-embedded as compared to acetone-fixed frozen sections. MoAb that were found to stain granular in the Golgi region of mucous cells (HH5, BM8, TKH6, B72.3, and TKH2) deviated only in intensity, with brighter staining of frozen sections. MoAb that were found in frozen sections to produce a diffuse staining of the cytoplasm of duct, serous, or myoepithelial cells (HH8, MBr1 and HH14) did not stain paraffin sections at all whether these were pretreated with neuraminidase or not, except HH8 that, after this pretreatment, produced a granular staining of serous acini. The following therefore only describes data on frozen sections.

Duct Cells: Duct cells were stained homogeneously in cytoplasm by MoAb to T (HH8) (Fig 5*a,d*) and mucin-type H (MBr1 and HH14) (Fig 5*b,e*) irrespective of ABO blood group and secretor status. The intensity of the two anti–mucin-type H MoAb varied as also seen in stratified epithelium. HH14 labeled fewer cells than MBr1. No staining was observed with MoAb to mucin-type A and Tn/sialosyl-Tn.

Mucous Cells: No staining was observed with the MoAb to T (HH8) with or without neuraminidase treatment of sections. Anti–mucin-type H MoAb (MBr1, HH14) stained diffusely in the area of the nucleus of some cells of secretor individuals, whereas other cells were negative (Fig 5*b*).

The MoAb to mucin-type A (HH5) stained granular in the basal supra nuclear area, suggesting a localization to the Golgi apparatus. A similar staining pattern was previously observed with MoAb to the histo-blood group A glycosyltransferase that generally is believed to be located in the Golgi membrane [44] (Fig 5*c*). Similar to MBr1, HH5 gave a patchy distribution of positively stained cells, intermixed with negative cells. Mucin-type A was found only in secretor individuals of A_1 as well as A_2 subgroup status, with no apparent consistent difference between the subgroup in contrast to the staining observed in buccal mucosa.

MoAb to Tn (BM8, TKH6) (Fig 6*a*) and sialosyl-Tn (B72.3, TKH2) (Fig 6*b*) stained granular in the basal nuclear area similar to HH5, although B72.3 and TKH2 stained considerably weaker. The expression of Tn and sialosyl-Tn was independent of blood group and secretor status.

Serous Cells: No staining was observed with the anti-T MoAb HH8, but pretreatment of sections (both frozen and paraffin embedded) with neuraminidase produced a granular staining with different intensity varying from hardly detectable to bright (not shown). Anti–mucin-type H MoAb MBr1 and HH14 stained the cytoplasm homogeneously in all blood groups of secretors but not in nonsecretors, in contrast to our finding that duct cells stained in both groups (Fig 5*b,e*). No staining was found with MoAb to mucin-type A and Tn/sialosyl-Tn.

Myoepithelial Cells: These were labeled by the anti-T MoAb (HH8), and pretreatment of sections with neuraminidase did not change this pattern (Fig 5*a,d*). To further substantiate this, we performed duplicate stainings with MoAb HH8 and anti-α and γ vascular smooth-muscle actin MoAb HHF-35, which identifies myoepithelial cells of salivary glands [45] (Fig 7). HH8 showed a reaction similar to the actin MoAb in the location of myoepithelial cells. Labeling of myoepithelial cells by HH8 was found in all blood groups of secretors and nonsecretors. Myoepithelial cells were not stained by any of the other anti–mucin-type MoAb tested.

DISCUSSION

In the present study we have analyzed the simple mucin-type carbohydrate expression in oral epithelia using a comprehensive panel of MoAb (Table II). In Fig 1 the present concept of mucin-type glycosylation is depicted. The panel of MoAb used in this study allows detection of most of the carbohydrate structures formed during biosynthesis of the simple mucin-type glycans in man. The special interest in simple mucin-type glycans is due to the rather general cancer-associated expression of the most immature structures Tn, sialosyl-Tn, and T, as found in numerous studies [1]. The specificity of the MoAb used generally allows for structural interpretation of reacting tissue antigens. Three of the MoAb (HH8, MBr1, and HH5) react with carbohydrate structures on lipids, in addition to the mucin-type structures on proteins [34]. The MoAb HH8 reacts with a histo-blood group A-associated glycolipid not present in O or B

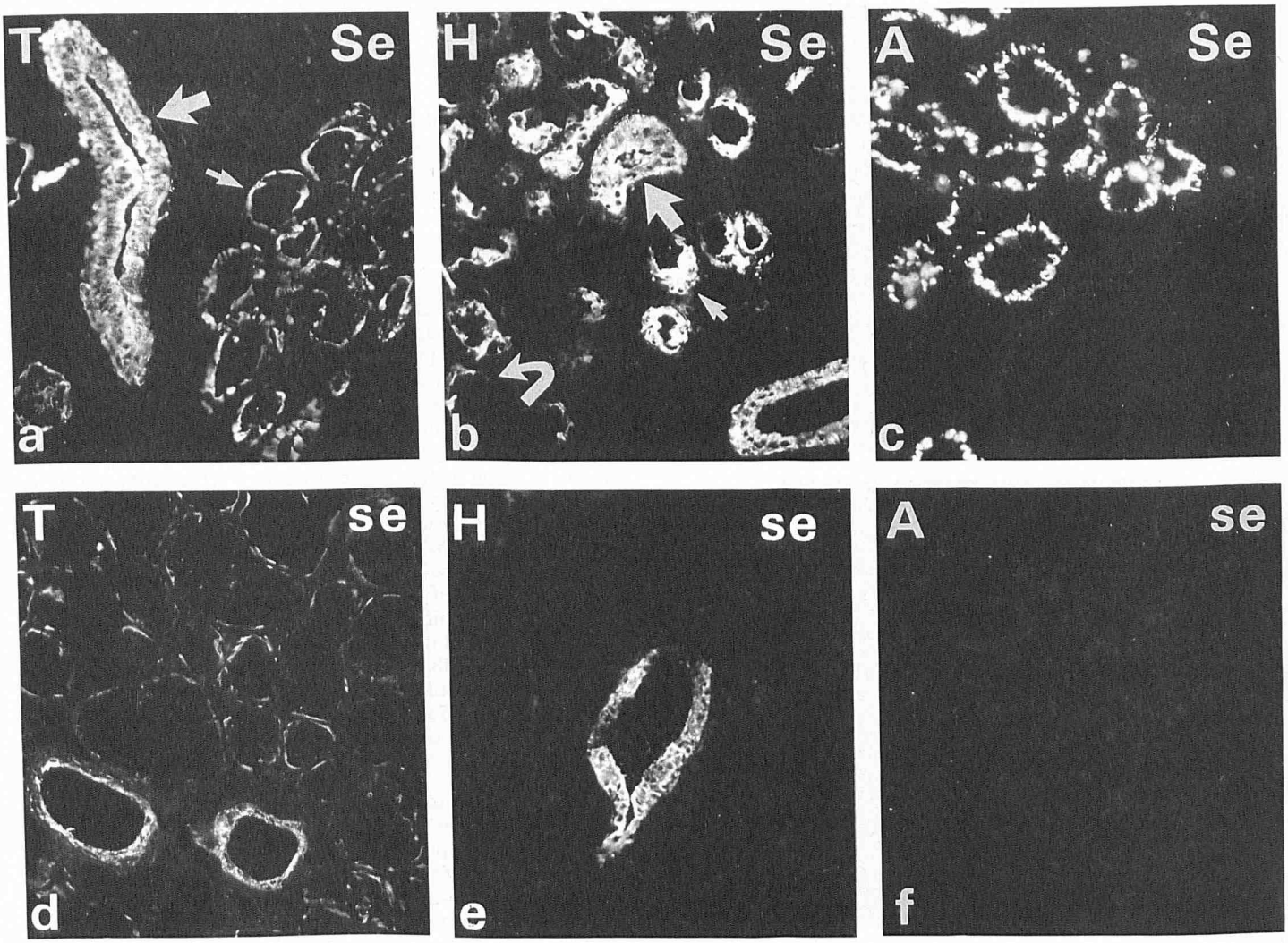

Figure 5. Immunohistologic staining of human oral small salivary glands. Simple mucin type antigens in Le^{a-b+}, secretor individuals (Se) *(a,b,c)* and in Le^{a+b-}, nonsecretor individuals (se) *(d,e,f)*. *(a,d)* Similar distribution of T in duct *(large arrow)* and myoepithelial *(small arrow)* cells in secretor and nonsecretor. (Blood group O individuals stained with MoAb HH8, ×120.) *(b)* H in duct *(large arrow)* and serous *(small arrow)* cells and in basal nuclear region of mucous cells *(bent arrow)* in secretor versus staining of only ductus cells in non-secretor (O individuals stained with MoAb MBr1, ×120). *(c)* A in basal nuclear area of mucous cells in secretor (A_1 individual) versus *(f)* no staining of nonsecretor (A_2 individuals stained with MoAb HH5, ×120).

individuals apart from the T antigen, but as shown here HH8 stains ABO tissues similarly, indicating that the antigen detected is mucin-type T. Our findings that 1) these MoAb stained fixed paraffin-embedded and deparaffinized sections, a procedure believed to remove at least some glycolipid structures [37], and 2) a reciprocal relationship between the staining of HH8 and MBr1 in stratified epithelium of secretors versus nonsecretors was observed indicate that the antibodies indeed are detecting simple mucin-type variants.

In previous studies we have shown that cellular maturation in stratified epithelium is characterized by a step-by-step build-up of carbohydrate chains of poly-N-acetyllactosamine structures in accordance with the biosynthetic pathway [46,47]. Basal cells are characterized by unsubstituted N-acetyllactosamine (Galß1 → 4GlcNAcß1-R), whereas cells immediately above express the fucosylated derivative H (Fucα1 → 2Galß1 → 4GlcNAcß1-R), and finally the rest of the spinous cell layers were found to express the difucosylated derivative Le^y [48] as well as blood group A and B variants hereof in the appropriate blood groups.

The simple mucin-type glycosylation studied in the present paper showed similar sequential expression in relation to the maturation process in stratified epithelium. This suggests that the sequential synthesis of carbohydrate structures as cells move through the epithelium is controlled by the enzymes involved in the glycosylation process, i.e., the fucosyltransferase and the A or B transferases. Recently, we have found that this in fact is the case for the histo-blood group A transferase, where MoAb to the A transferase protein stained the same mature cell layers as did MoAb to the A carbohydrate antigen, indicating that the appearance of A carbohydrate antigens in superficial layers is directly controlled by the A gene and its primary product [44].

Cells close to the basal membrane that are considered the most immature cells of the stratified epithelium were found to express sialosyl-T, whereas more mature cells in cell layers above produced more complex mucin-type structures. In this context one would have expected that the more immature Tn and sialosyl-Tn structures would be expressed by immature cells, or not be present at all. However, in contrast, Tn and sialosyl-Tn antigens were seen in the superficial cell layers, but as a weak granular cytoplasmic staining. Because the superficial cells expressing these immature carbohydrate structures in the cytoplasm also express the fully mature products, H and A mucin-type structures, it is conceivable that cytoplasmic accumulation of Tn and sialosyl-Tn is the result of arrested glycosylation with intracellular (Golgi) accumulation of truncated glycoproteins. This interpretation appears likely when considering

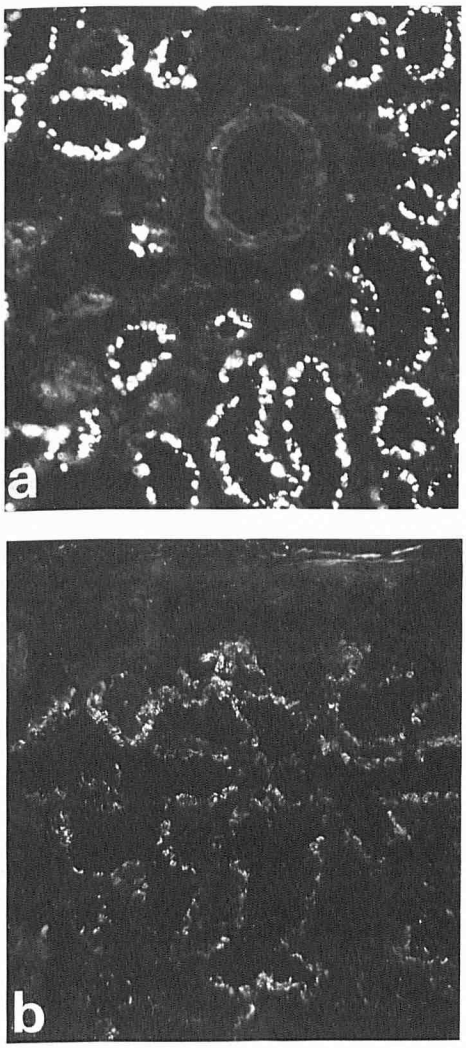

Figure 6. Immunohistologic staining of human oral small salivary glands. *(a)* Tn stained with MoAb BM8 in basal nuclear area of mucous cells (blood group O, Le^{a+b-}, secretor individual × 120) and *(b)* sialosyl-Tn stained with MoAb B72.3 in same location as Tn, although weaker staining (same individual).

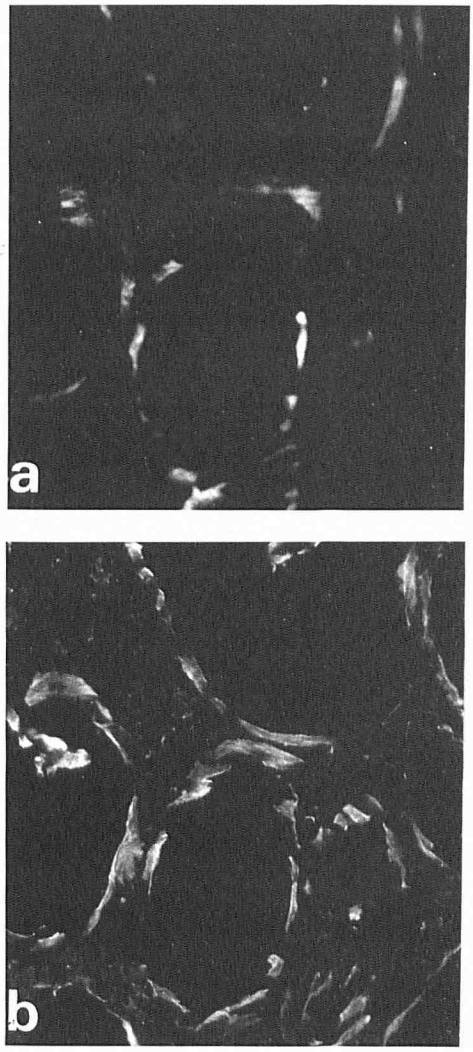

Figure 7. Double immunohistologic staining of myoepithelial cells in human minor salivary glands. Anti-T MoAb HH8 *(a)* in same cells that stain with anti-α and γ vascular smooth-muscle actin MoAb HHF-35 *(b)*.

the nature of the terminal differentiation process of stratified epithelium in which superficial cells are destined to die and exfoliate. Tn and sialosyl-Tn have, however, been found on the cell membrane of stratified epithelia in conjunction with hyperproliferative lesions such as candidal hyperplasias in oral epithelium, psoriatic lesions, and "normal" epithelium adjacent to carcinomas [49,50]. Therefore, an alternative interpretation may be that a generally increased biosynthetic activity leads to an increase in precursor structures prone to further modifications.

The interesting observation that on occasion single cells in the basal cell layer at the base of rete pegs of the stratified epithelium were found to label unsubstituted T in frozen sections of labial mucosa deserves further work. The labeled cells may represent a subset of less mature cells compared to the other immature cells of this layer, or these single cells could be non-epithelial. Preliminary attempts to clarify if these cells were indeed keratinocytes using staining of serial sections with antibodies to keratin 18, 19 (Merckel cells), vimentin, and OKT8 (lymfocyte subsets) (not shown) did not indicate a specific nonkeratinocyte cell, and thus it may presently not be ruled out that these cells are indeed keratinocytes.

The Tn and sialosyl-Tn antigens are only very limited expressed in normal adult tissues; when present they are occluded by covering structures and therefore inaccessible to the immune system. All humans have pre-existing antibodies against T and Tn and these were demonstrated to be elicited by the intestinal flora [1]. In this study these were found in both stratified and glandular normal adult epithelia, but the expression was mainly found intracellularly, in contrast to other carbohydrate antigens. Based on comparison with immunocytolocalization of the histo-blood group A transferase [44], it is likely that the staining is associated with the Golgi apparatus. The cytolocation of these antigens could be a result of temporary accumulation of biosynthetic precursors in the Golgi before further processing, or simply that Golgi-associated proteins are glycosylated this way. Alternatively, as discussed in the superficial cell layers of stratified epithelia arrested metabolism/glycosylation could account for this location.

The finding that the simple mucin-type carbohydrate structures were almost exclusively expressed close to the nucleus in the Golgi area but not in the general cytoplasm/secretory vesicles and in cell membranes of the salivary gland mucous cells is in agreement with absence of these structures in saliva. This is in striking contrast to immunocytolocalization of N-acetyllactosamine based structures (type 1 and 2 chain), Lewis and ABH related structures, which are found in the entire cytoplasm, i.e., secretory vesicles, of mucous cells [48,51–54]. Chemical analysis of saliva glycoproteins (mucins) have found only complex mucin-type glycosylation,

where the Tn or T core is substituted with N-acetyllactosamine chains mainly of type 1 chain endings (see Fig 1) [34]. The immunohistologic staining of acini cells therefore correlates well with the composition of saliva mucins.

The distribution of mucin-type antigens in minor salivary glands may reflect the different cell functions [43]. Contractile myoepithelial cells expressed immature T and nonsecreting duct cells expressed T and the more mature mucin-type H. Serous cells that predominantly secrete glyco-proteins expressed mucin-type H and sialosyl-T, whereas mucous cells rich in secretory granules and mainly secreting polysaccharides expressed mucin-type H and the mature mucin-type A structure. In addition mucous cells expressed immature Tn and sialosyl-Tn. The finding that myoepithelial cells are labeled by anti-T MoAb HH8 is interesting as a putative marker because these cells are numerous in salivary gland tumors and may play an important part of tumor genesis [45,55].

Previous studies using lectins do not correlate well with the present data, but this may be attributed to the more broad reaction pattern of lectins as compared to MoAb [21,56]. PNA-lectin that preferably reacts with T-antigen was observed in basal cells of oral mucosa where we found sialosyl-T [57,58]. PNA has been found to stain serous cells [59] and mucous cells of small salivary glands [60] in contrast to our finding that T antigen was found in duct and myoepithelial cells. Furthermore, lectins used to detect the Tn antigen have been shown to stain in the Golgi region of acini similar to our results, but in addition staining was found of secretory granules [59,60].

The results obtained in this study is considered of value as a baseline for studies of simple mucin-type glycosylation in premalignant and malignant disorders of stratified oral epithelium. Stratified epithelium of the oral cavity in contrast to epithelium of salivary glands utilize the simple mucin-type glycosylation of membrane proteins, i.e., sialylated-T and the ABH derivatives are expressed at the cell membrane. Incomplete synthesis of mucin-type glycosylation in these cells in association with malignant transformation may therefore result in the accumulation of the biosynthetic precursors T, Tn, and sialosyl-Tn. In a recent study we found changed sequential expression of simple mucin-type antigens in premalignant lesions as compared to normal [61]. The Tn, sialosyl-Tn, and T antigens therefore appear to be good candidates as markers of disorders in the differentiation of oral epithelia. These antigens are especially valuable in this context because they are not subject to individual to individual (intraspecies) genetic variability, although build-up of these core structures are, e.g., ABH polymorphism as shown here.

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